

Virulence of *Leishmania major* in macrophages and mice requires the gluconeogenic enzyme fructose-1,6-bisphosphatase

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Leishmania are protozoan parasites that replicate within mature phagolysosomes of mammalian macrophages. To define the biochemical composition of the phagosome and carbon source requirements of intracellular stages of *L. major*, we investigated the role and requirement for the gluconeogenic enzyme fructose-1,6-bisphosphatase (FBP). *L. major* FBP was constitutively expressed in both extracellular and intracellular stages and was primarily targeted to glycosomes, modified peroxisomes that also contain glycolytic enzymes. A *L. major* FBP-null mutant was unable to grow in the absence of hexose, and suspension in glycerol-containing medium resulted in rapid depletion of internal carbohydrate reserves. *L. major* Δfbp promastigotes were internalized by macrophages and differentiated into amastigotes but were unable to replicate in the macrophage phagolysosome. Similarly, the mutant persisted in mice but failed to generate normal lesions. The data suggest that *Leishmania* amastigotes reside in a glucose-poor phagosome and depend heavily on nonglucose carbon sources. Feeding experiments with [^{13}C]fatty acids showed that fatty acids are poor gluconeogenic substrates, indicating that amino acids are the major carbon source *in vivo*. The need for amino acids may have forced *Leishmania* spp. to adapt to life in the mature phagolysosome.

gluconeogenesis | intracellular pathogens | phagolysosome | carbohydrate | mannan

Leishmania are parasitic protozoa that cause a spectrum of important diseases in >12 million people worldwide, ranging from self-healing cutaneous lesions to nonhealing mucocutaneous and visceral disease. There are no effective vaccines against leishmaniasis, and current drug treatments are characterized by low efficacy, high toxicity, and expense or, in the case of front-line antimonial drugs, widespread resistance (1). Infection of humans and other animal hosts is initiated by flagellated promastigotes that develop within the digestive tract of the sandfly vector and are injected into the skin during a sandfly blood meal. Promastigotes are internalized into mature phagolysosomes of a number of phagocytic host cells, including neutrophils, dendritic cells, and macrophages but proliferate only within the latter (2). The proliferative intracellular stages are aflagellate amastigotes that remain within the phagolysosome and perpetuate infection in the host through repeated cycles of macrophage infection.

Very few other microbial pathogens are capable of proliferating within macrophage phagolysosomes (3), and little is known about the nutrient composition of this compartment or the metabolic responses of persistent *Leishmania* amastigote stages (4). Glucose uptake appears to be essential in promastigote infection of macrophages, because promastigotes of a *Leishmania mexicana* mutant lacking all glucose transporters are unable to infect macrophages (5). However, this mutant fails to differentiate into amastigotes *in vitro* (5), and the hexose requirements of this stage are largely unknown. Glucose and other hexoses are

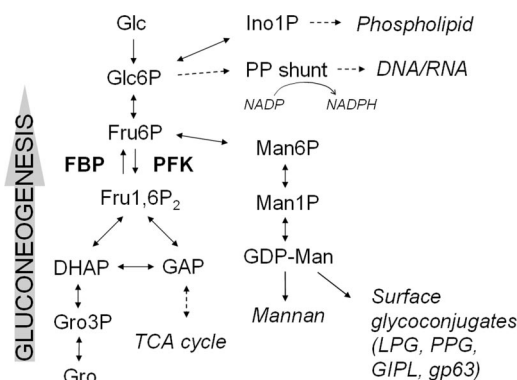


Fig. 1. Hexose metabolism in *L. major*. Hexoses are required for glycolysis, the pentose phosphate shunt (PP-shunt), and the biosynthesis of intracellular carbohydrates (β 1,2-mannan), inositolphospholipids, and surface glycoconjugates, such as glycoinositolphospholipids (GIPL), lipophosphoglycan (LPG), proteophosphoglycan (PPG), and gp63 glycoprotein. DHAP, dehydroxyacetonephosphate; Fru6P, fructose-6-phosphate; Fru1,6P₂, fructose-1,6-bisphosphate; Glc6P, glucose-6-phosphate; GAP, glyceraldehyde-3-phosphate; Gro3P, glycerol-3-phosphate; Ino1P, inositol-1-phosphate; Man1P, mannose-1-phosphate; Man6P, mannose-6-phosphate. FBP and phosphofructokinase (PFK) catalyze key reactions in gluconeogenesis and glycolysis, respectively.

a potential source of energy and are required to fuel the pentose phosphate pathway that maintains parasite redox balance and generates precursors for DNA and RNA biosynthesis (6). *Leishmania* also incorporate hexoses into β 1,2-mannan reserve oligosaccharides (7) and a number of complex surface glycoconjugates (7–10) that may be required for intracellular survival of promastigotes and amastigotes (Fig. 1). In this study, we have investigated whether the intracellular stages of *L. major* obtain all of their hexose requirements from the phagolysosome or whether they are dependent on *de novo* synthesis of hexose via gluconeogenesis. An *L. major* mutant lacking the enzyme fructose-1,6-bisphosphatase (FBP) that catalyzes the final committed step in gluconeogenesis (Fig. 1) was generated. Analysis of this mutant indicates that the obligate intracellular amastigote stages depend heavily on gluconeogenesis and nonglucose carbon sources, most likely amino acids, for *de novo* synthesis of essential cellular carbohydrates.

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Abbreviations: CDM, completely defined medium; FBP, fructose-1,6-bisphosphatase; HPAEC, high-pH anion-exchange chromatography.

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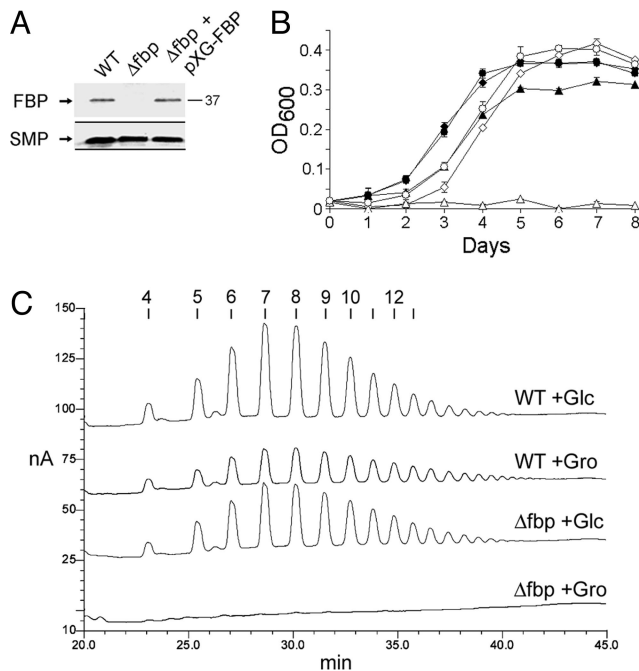


Fig. 4. Characterization of the *L. major* Δfbp mutant. (A) Western blot analysis of total protein from WT, Δfbp , and Δfbp complemented with pXG-FBP promastigotes. Blots were probed with anti-FBP antibody and the flagellum protein SMP1 (loading control). (B) WT (diamonds), Δfbp (triangles), or complemented Δfbp (circles) promastigotes were grown in CDM containing either glucose (filled symbols) or glycerol (open symbols) as the major carbon source. Cell growth was followed by measuring absorbance at 600 nm (OD_{600}). Standard deviations are derived from three separate cultures. (C) Cellular levels of intracellular $\beta 1,2$ -mannan oligosaccharides. Mannan was extracted from WT and Δfbp mutant promastigotes grown in glucose or glycerol and analyzed by HPAEC. The elution position of $\beta 1,2$ -mannan oligomers containing 4–13 mannose residues is indicated.

Growth Characteristics and Phenotype of a *L. major* FBP-Null Mutant.

An *L. major* FBP-null mutant (Δfbp) was generated by targeted replacement of the two chromosomal FBP alleles with bleomycin- and puromycin-resistant cassettes (see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site). Parasites resistant to both drugs were readily isolated, and correct insertion of the constructs and loss of the endogenous gene were confirmed by PCR (data not shown). The null mutant had <2% of the FBP activity of WT parasites (Fig. 2C) and lacked detectable levels of FBP protein by Western blotting (Fig. 4A). WT levels of FBP expression were restored by transfecting the null mutant with the pXG-vector containing the coding sequence for FBP (Fig. 4A).

Δfbp *L. major* promastigotes grew at a rate similar to that of WT promastigotes in completely defined medium (CDM) containing glucose (Fig. 4B). However, no growth was observed when Δfbp promastigotes were diluted in medium containing glycerol as the major carbon source (Fig. 4B). Complementation of the Δfbp mutant with a plasmid encoding FBP (Fig. 4B) or GFP-FBP (data not shown) completely restored the ability of the mutant to grow on glycerol. WT *L. major* promastigotes accumulate high levels of intracellular $\beta 1,2$ -mannan when cultivated in synthetic medium containing either glucose or glycerol as carbon sources (Fig. 4C). In contrast, the Δfbp mutant synthesized mannan only when provided with glucose, and these oligosaccharides were rapidly depleted when Δfbp was suspended in glycerol-containing medium (Fig. 4C). The cellular levels of major cell-surface glycoconjugates also decreased but at a slower rate than mannan (data not shown). These results show

Table 1. Utilization of [^{13}C]fatty acids by *L. major* promastigotes

Metabolite	+AA	–AA
Fatty acids		
C16:0	37	55
C18:0	50 (44)	22 (14)
C18:1	77 (63)	46 (32)
C18:2	70 (12)	48 (3)
TCA		
Malate	<5	ND
Fumarate	<5	ND
Sugars		
Glc-6-PO ₄	<5	ND
Fru-6-PO ₄	<5	ND

Parasites were cultivated in the presence of [^{13}C]fatty acids in glucose-free CDM with (+AA) or without (–AA) amino acids. Values refer to mol percent of each metabolite that was enriched in ^{13}C substituents. Numbers in parentheses refer to mol percent of C18 fatty acids that contained 14 or 16 ^{13}C substituents (M14, M16 mass isotopomers) and are, therefore, derived from exogenous C14 and C16 fatty acids. ND, not determined because of very low levels of these intermediates in amino acid-starved parasites.

that gluconeogenesis is capable of supplying all of the hexose requirements of *L. major* promastigotes.

Leishmania can use amino acids as carbon sources for gluconeogenesis (5). To investigate whether they can also use fatty acids, *L. major* WT promastigotes were cultured in glucose-free medium containing amino acids and [^{13}C]fatty acids. The major saturated (C16:0, C18:0) and unsaturated (C18:1, C18:2) fatty acids were labeled with ^{13}C (37–77%), and mass isotopomer analysis indicated that up to 63% of the C18:1 was synthesized by elongation of exogenous C14 and C16 fatty acids (Table 1). Uptake and *de novo* lipogenesis were reduced but still observed in parasites suspended in medium lacking both glucose and amino acids (Table 1). However, ^{13}C enrichment in tricarboxylic acid cycle intermediates and sugar phosphates was not detected under either glucose- or amino acid-starvation conditions (Table 1). These data suggest that, in contrast to the situation in many bacterial and fungal pathogens (15, 16), acetyl-CoA generated by fatty acid β -oxidation does not fuel gluconeogenesis in *L. major*.

Amastigotes of the Δfbp Mutant Are Unable to Replicate in Macrophages or Mice.

To investigate whether FBP and gluconeogenesis are required for parasite growth in macrophages, BALB/c bone-marrow-derived macrophages were infected with stationary-phase WT and Δfbp promastigotes. Both parasite lines expressed WT levels of surface glycoconjugates and mannan at the beginning of the infection (data not shown), infected macrophages to similar levels (Fig. 5A), and differentiated into amastigotes (Fig. 5B). However, Δfbp parasites failed to replicate in macrophages, whereas WT and complemented Δfbp parasites underwent at least two replications over 7 days (Fig. 5C). The Δfbp amastigotes remained viable but contained greatly reduced levels of intracellular mannan (Fig. 5D), suggesting that Δfbp amastigotes are hexose-limited. These data suggest that amastigotes can scavenge some sugars from the phagolysosome but that these pools are insufficient to sustain parasite growth in the absence of gluconeogenesis.

To investigate whether FBP and gluconeogenesis are also required for proliferation of *Leishmania* amastigotes in animals, susceptible BALB/c mice were infected with stationary-phase promastigotes. WT and complemented Δfbp parasites induced lesions between 2 to 4 weeks that continued to grow over 12 weeks. In contrast, Δfbp parasites failed to induce mature lesions over 27 weeks (Fig. 6), although the majority of the mice (7 of 11) developed small s.c. swellings at the site of injection that contained parasites by histology (Fig. 6) (data not shown).

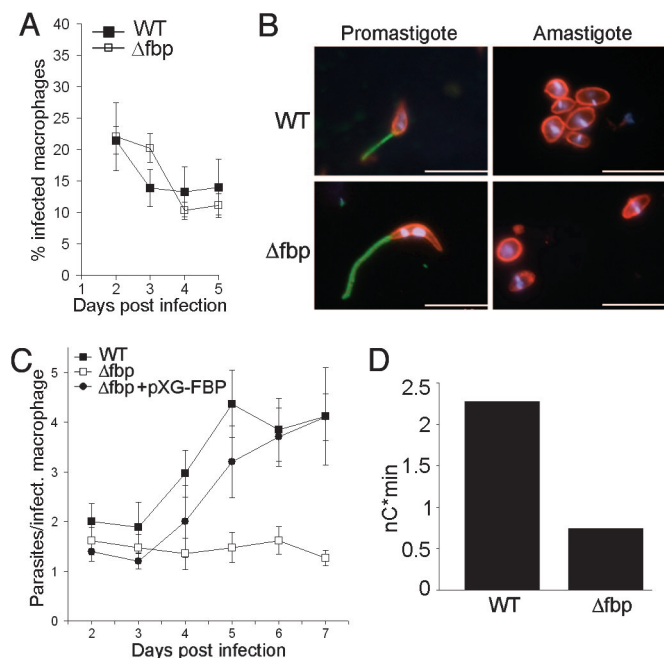


Fig. 5. The *L. major* Δfbp mutant is unable to replicate in macrophages. (A) Infection of BALB/c bone-marrow-derived macrophages with WT and Δfbp promastigotes. (B) Both WT and Δfbp promastigotes differentiated to small spherical amastigotes after internalization in macrophages. Amastigotes extracted from macrophages (day 5) were fixed and labeled with antibodies to the flagellum protein SMP-1 (green), α -tubulin (red), and Hoechst (blue). (C) Replication of WT, Δfbp , and pXG-FBP-complemented Δfbp in bone-marrow-derived macrophages. Samples were analyzed in triplicate from two independent experiments ($n = 300$ macrophages; error bars indicate SD). (D) Mannan levels in WT and Δfbp amastigotes (10^6 cell equivalents) were detected by HPAEC (arbitrary conductivity units).

Parasites deficient in gluconeogenesis are, thus, able to persist in macrophages and susceptible animals but are unable to proliferate at a rate sufficient for lesion development.

Discussion

Most microbial pathogens that invade and proliferate within macrophages avoid mature phagolysosomes or rapidly escape from this compartment (3). *Leishmania* spp. are unusual in having the capacity to both survive and proliferate within this compartment. Here, we show that gluconeogenesis is important for amastigote replication in macrophage phagolysosomes and for proliferation in susceptible animals. Our findings suggest that the *L. major*-occupied phagolysosomes are hexose-poor and that

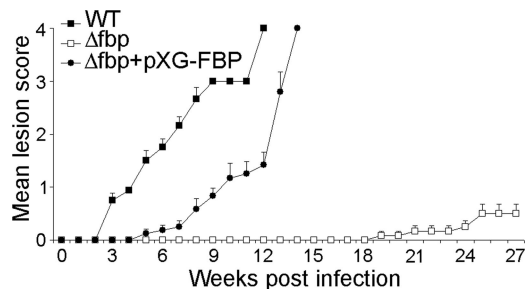


Fig. 6. The *L. major* Δfbp mutant can persist but does not cause pathology in mice. BALB/c mice were infected intradermally with 10^6 stationary-phase promastigotes. Lesion formation and progression were monitored weekly. Error bars represent SD. Lesion results are representative of two independent experiments.

amastigotes scavenge other carbon sources for energy and the synthesis of essential carbohydrates.

Leishmania parasites depend on carbohydrates to sustain central carbon metabolism (glycolysis and pentose phosphate pathways) and the biosynthesis of abundant classes of surface (glyco)lipids, glycoproteins, proteoglycans, and intracellular oligosaccharides (6, 7, 9, 10, 17) (Fig. 1). Extracellular promastigote appear to depend on uptake of exogenous hexose *in vivo*, because promastigotes of the *L. mexicana* glucose transporter are less virulent than WT parasites in the sandfly vector and completely avirulent in macrophages (5). Because this mutant fails to differentiate into amastigotes in the macrophage, it remains unclear whether hexose uptake is also essential for intracellular growth of the latter stage (5). In contrast, *L. major* Δfbp promastigotes invade macrophages and differentiate into amastigotes with the same kinetics as WT parasites. Although the Δfbp amastigotes remain viable, they are unable to replicate in the macrophage and contain very low levels of reserve mannan. These data suggest that macrophage phagolysosomes contain low levels of sugars and that gluconeogenesis is required to supplement or provide most of the hexose requirements of *L. major* amastigotes. Gluconeogenesis also appears to be up-regulated in *L. mexicana* amastigotes (M.E. and M.J.M., unpublished data), coincident with a marked down-regulation in glucose transport activity in amastigotes of this and other species (18), suggesting that gluconeogenesis is important for intracellular growth of all *Leishmania*. These findings imply that *Leishmania* amastigotes will depend highly on tricarboxylic acid cycle reactions and respiration for energy generation rather than glycolysis.

Gluconeogenesis is important for intracellular survival of several bacterial and fungal pathogens that replicate within various vacuolar compartments in macrophages (15, 19, 20). However, in contrast to *L. major*, most of these pathogens can use fatty acids as their major carbon source and channel acetyl-CoA, the major product of fatty acid β -oxidation, into gluconeogenesis via the glyoxylate pathway (15, 16, 20). Although *L. major* parasites have the capacity to take up and catabolize fatty acids (18), [^{13}C]fatty acid labeling experiments indicated that the products of β -oxidation are not used for gluconeogenesis, even when the parasites are glucose-starved. These data are consistent with the finding that the *L. major* genome lacks homologues for the two glyoxylate shunt enzymes, isocitrate lyase and malate synthase, needed for net synthesis of sugars from acetyl-CoA (21), despite early studies suggesting that these enzymes were present (22). *Leishmania* may, thus, depend heavily on exogenous or endogenous pools of amino acids for gluconeogenesis. In support of this notion, (i) the *L. major* genome encodes for a large number of putative amino acid permeases and the enzymatic machinery needed to catabolize and divert the carbon backbones of amino acids into gluconeogenesis (23), (ii) the activity of some amino acid permeases is up-regulated in isolated amastigotes (24), and (iii) growth of *Leishmania* amastigotes in macrophages and animals depends on lysosomal hydrolases that generate an important source of amino acids for metabolism (25, 26). The need to obtain essential amino acids (21) and nonfatty acid carbon sources for gluconeogenesis may have forced *Leishmania* amastigotes to adapt to life in the hydrolytic but amino acid-rich phagolysosomes (2), rather than earlier, less hydrolytic compartments in the phagocytic pathway. Intriguingly, other pathogens that reside long term in the macrophage phagolysosome, such as the Gram-negative bacterium *Coxiella burnetii* also lack the glyoxylate shunt enzymes and depend on high levels of amino acids for growth (27). Finally, the nutritional requirements of *Leishmania* amastigotes may explain why these parasites fail to replicate in other phagocytic cells, such as neutrophils and dendritic cells, that are thought to have amino acid-poor phagosomes (2, 28).

Our results indicate that the differentiation of *Leishmania* promastigotes to amastigotes in the macrophage is associated with marked changes in flux through the glycolytic and gluconeogenic pathways. However, this differentiation step is not associated with clear changes in mRNA and protein levels of any glycolytic/gluconeogenic enzymes (ref. 29 and this study) and many of the enzymes in these opposing pathways are sequestered within the same subcellular compartment (ref. 30 and this study). Well characterized allosteric regulators of glycolysis and gluconeogenesis, such as fructose-2,6-biphosphate, are apparently excluded from glycosomes (31), and little is known about the regulation of these pathways in trypanosomatids. We speculate that the well characterized down-regulation of the glucose transporter activity in *Leishmania* amastigotes (18) may facilitate the switch from glycolysis to gluconeogenesis in this stage. Our findings suggest that inhibitors of gluconeogenesis may be useful in preventing growth of intracellular *Leishmania* and that gluconeogenic mutants, such as Δfbp , that persist but are poorly proliferative in macrophages, constitute a useful platform for development of attenuated live vaccines (32–34).

Materials and Methods

Parasite Strains, Plasmids, and Cell Culture. Promastigotes of *L. major* (MHOM/SU/73/5ASKH) were cultured either in semidefined medium-79 (SDM) supplemented with 10% FBS or in CDM (35) at 27°C. For isolation of transfected parasites, media were supplemented with G418 (100 μ g/ml), puromycin (20 μ g/ml), and/or bleomycin (10 μ g/ml) and colonies isolated from SDM-agar plates. For expression of the *L. major* FBP in the FBP-deficient *E. coli* strain DF657 (*E. coli* Genetic Stock Center, Yale University, New Haven, CT) (13), the FBP ORF was amplified with primers 704 (CGGGATCCATGGACGTCA-GACGCACCCC) and 705 (CGGAATTCTTAGAGCTTGCT-GGAAGTCGG) and cloned into the BamHI and EcoRI sites of pGEX-6P3 (GE Healthcare). The restriction sites are underlined. Bacteria were transformed with either the pGEX vector or the pGEX vector containing *L. major* FBP. The *L. major* Δfbp mutant was constructed by sequential replacement of the two FBP alleles with bleomycin- and puromycin-resistance cassettes, as described in *Supporting Materials and Methods*.

Western Blotting Analysis. Parasite lysates were analyzed by 12% SDS/PAGE (10⁷ parasite equivalents per lane) and Western blotting (14). Membranes were probed with anti-GFP (Roche) or anti- α -tubulin (Sigma) monoclonal antibodies (1:1,000 dilution) or with anti-*L. major* FBP (1:1,000 dilution) and anti-*Trypanosoma brucei* BiP (1:2,000 dilution) polyclonal antibodies. The anti-FBP polyclonal antibody was generated against a 17-aa peptide (*Supporting Materials and Methods*) conjugated to tetanus toxin (AusPep, Parkville, Victoria, Australia). The conjugate was used to immunize New Zealand White rabbits in Freund's complete adjuvant. Rabbits were boosted three times at four weekly intervals, and antibodies were purified from whole sera by using a peptide-affinity column.

Subcellular Localization of FBP. Live *L. major* promastigotes expressing GFP constructs were harvested by centrifugation (800 \times g for 10 min at 25°C), resuspended in PBS, and overlaid on poly-L-lysine-coated coverslips. Immobilized promastigotes were mounted with Hoechst 33342 (Molecular Probes) containing Mowiol 4-88, and images were acquired by using a Zeiss Axio-plan2 imaging microscope, equipped with AxiCam MRm camera and the AXIOVISION 4.3 software (Zeiss). Subcellular fractionation was performed after hypotonic lysis of *L. major* promastigotes expressing GFP-FBP and isopycnic velocity centrifugation of a postnuclear fraction (500 \times g supernatant) in a

15–80% sucrose gradient (14). Protein concentration in each fraction (700 μ l) was measured by using the BCA assay (Pierce). Organelle markers were detected after SDS/PAGE and Western blotting with polyclonal antibodies against *T. brucei* BiP (1:2,000 dilution), *L. mexicana* phosphomannose mutase (1:1,000 dilution) (36), or *L. major* FBP (1:1,000 dilution). Hexose kinase activity was quantitated by following the formation of NADH (OD₃₄₀) in 75 mM triethanolamine, 10 mM glucose, 0.1% Triton X-100, 2.5 mM MgCl₂, and 1.5 mM NAD⁺, after addition of *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (1 unit; Sigma) and 1 mM ATP.

Analysis of FBP Activity and Expression of Cellular Glycoconjugates.

Promastigotes were suspended in hypotonic buffer (10 mM Hepes buffer, pH 7.4, 2 mM EGTA, and 2 mM DTT) and lysed by syringing, and the membrane fraction obtained after centrifugation (3,000 \times g for 10 min at 0°C) was suspended in 50 mM Hepes, pH 7.4, 2 mM EGTA, 5 mM MgCl₂, 1 mM MnCl₂, 1 mM DTT, protease inhibitor mixture, and 0.1% Triton X-100. FBP activity in the lysate (10⁶ cell equivalents in 100 μ l) was measured by addition of 10 nmol of FBP and incubation at 27°C for 10 min. The reaction was stopped, and products were extracted in chloroform/methanol/water (1:2:0.8 vol/vol). The supernatant was dried under nitrogen, and metabolites were partitioned in 1-butanol/water (2:1 vol/vol). The aqueous phase was dried and analyzed by high-pH anion-exchange chromatography (HPAEC) on a Dionex BioLC system equipped with a pulse amperometric detector (37). Cellular levels of the intracellular mannan were determined by HPAEC as described in ref. 7.

[¹³C]Fatty Acid Labeling. *L. major* promastigotes were cultivated in glucose-free CDM containing 200 μ M unlabeled fatty acids bound to 1% BSA (4 h), then suspended in glucose- or glucose/ amino acid-free CDM medium containing 200 μ M [¹³C-U]fatty acids (Spectra Stable Isotopes, Columbia, MD). After incubation for 24 h at 27°C, parasites were extracted in chloroform/methanol/water (1:2:0.8 vol/vol), and the supernatant was dried under nitrogen and partitioned between 1-butanol and H₂O (2:1 vol/vol). The organic phase was treated with methanolic 0.5 M HCl (80°C, 14 h) to release fatty acids as their methyl esters, whereas sugar phosphates and tricarboxylic acid cycle intermediates in the polar phase were methoximated and converted to their trimethylsilyl derivatives (38). The fatty acid methyl esters and polar metabolites were analyzed by GC-MS with an Agilent Technologies 6890/5973 system (7). Incorporation of [¹³C]fatty acids into parasite lipids or [¹³C]acetyl-CoA into polar intermediates was determined by mass isotopomer analysis of individual metabolite peaks (39).

Infection of Macrophages and Mice. Bone-marrow-derived macrophages from BALB/c mice (2 \times 10⁵ cells per ml) were grown on 10-mm coverslips in RPMI medium 1640 supplemented with 10% FBS, 4 mM glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 15% (vol/vol) L929 cell-conditioned media for 24 h at 37°C with 5% CO₂. Macrophage monolayers were overlaid with *L. major* promastigotes (1 \times 10⁶), grown to stationary phase in glucose-containing media without complement opsonization, and incubated for 4 h at 33°C in 5% CO₂. Coverslips were washed three times with PBS to remove unattached parasites and then incubated in fresh macrophage medium for up to 7 days at 33°C in 5% CO₂. Coverslips were washed three times in PBS and incubated subsequently in methanol (4°C, 10 min), PBS containing 50 mM NH₄Cl (4°C, 10 min), 0.05% saponin in PBS (4°C, 5 min), and 1% BSA in PBS (25°C, 30 min). The fixed cells were probed with mAb anti-LAMP-1 (1:800 dilution in 1% BSA in PBS; BD Biosciences) and Alexa Fluor-488 goat anti-rat (1:1,000 dilution; Molecular Probes) to visualize phagolysosomal membranes. Parasites were

detected with 0.2 $\mu\text{g}/\text{ml}$ propidium iodide and Hoechst by fluorescence microscopy. For isolation of intracellular amastigotes, infected bone marrow macrophages were suspended in chilled PBS containing 1 mM EDTA and 11 mM Glc and syringed five times with a 27-gauge needle. Cellular debris was removed by centrifugation ($60 \times g$ for 5 min) and passaged through a 3- μm pore filter, and amastigotes were recovered by centrifugation of the filtrate ($800 \times g$ for 10 min).

Female BALB/c mice (6–8 weeks old) derived from a specific pathogen-free facility were maintained under conventional conditions. Groups of mice (eight in each treatment) were inoculated intradermally at the base of the tail with 10^6 stationary-phase promastigotes (in 50 μl). Lesion development was monitored weekly by measuring the lesion diameter (40). All

studies were conducted in accordance with the Institutional Animal Care and Use Committee guidelines.

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